HOKKAIDO UNIVERSITY

Title	DEVELOPMENT OF MOUSE EMBRYONIC NUCLEI TRANSFERRED TO ENUCLEATED OOCYTES AND ZYGOTES
Author(s)	CHEONG, Hee Tae; TAKAHASHI, Yoshiyuki; KANAGAWA, Hiroshi
Citation	Japanese Journal of Veterinary Research, 40(4), 149-159
Issue Date	1992-12-28
DOI	https://doi.org/10.14943/jjvr.40.4.149
Doc URL	https://hdl.handle.net/2115/2406
Type	departmental bulletin paper
File Information	KJ00002377610.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Jpn. J. Vet. Res., **40**, 149–159 (1992)

DEVELOPMENT OF MOUSE EMBRYONIC NUCLEI TRANSFERRED TO ENUCLEATED OOCYTES AND ZYGOTES

Hee Tae CHEONG, Yoshiyuki TAKAHASHI, and Hiroshi KANAGAWA

(Accepted for publication: Sep. 19, 1992)

ABSTRACT

The present study was conducted to examine the development of nuclear transplant embryos produced by transplanting nuclei to either oocytes or zygotes in the mouse. Metaphase II oocytes and one-cell zygotes were enucleated and fused with transferred nuclei from late two-, four- and eight-cell stage embryos. Enucleation of metaphase oocytes was achieved using the interference microscope without staining. Fusion and oocyte activation were performed by means of electric fields. Similar development rates to the blastocyst stage were obtained from enucleated oocytes (28.0%) and zygotes (30.9%) reconstituted with nuclei from late two-cell embryos. Cleavage and blastocoele formation of reconstituted embryos occurred at around the same time as observed in the control embryos, with some exceptions. After transfer to recipient females, live young were obtained from both reconstituted oocytes (9.1%) and zygotes (11.5%) that received a nucleus from late two-cell embryos. The results indicate that enucleated zygotes as well as oocytes can support development to term of nuclei introduced from late two-cell embryos in which activation of the embryonic genome has occured, which may be a result of the reprogramming of the donor nucleus.

Key words: Nuclear transplantation, nuclear reprogramming, mouse

INTRODUCTION

Transplantation of a nucleus to a recipient cell involves the reconstitution of a nucleus and cytoplasm within different developmental stage. Therefore, for nuclear trasfer to succeed the transferred nucleus must be reprogrammed in its developmental schedule. Reprogramming involves the modification of the transferred nucleus so that it behaves as if it was a zygote nucleus, and that nuclear transfer embryos form a blastocoele at a time corresponding to a zygote and at normal cell number. It has

Department of Theriogenology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

been suggested that the ability to reprogram the transferred nuclei is restricted to the mature oocyte and is not present in the pronuclear stage embryo[19,20,23]. Transplantation of nuclei from cleavage-stage embryos to enucleated pronuclear stage zygotes resulted in very limited development in mice[15] and cattle[22]. However, enucleated oocytes receiving ICM cell nuclei[11] and male primordial germ cell nuclei[27] could develop to the blastocyst stage in the mouse. Furthermore, development to the blastocyst stage or viable offspring were obtained from enucleated oocytes receiving nuclei from eight- and 16-cell stage embryos in rabbits[4,5], eight- and 16-cell stage embryos or ICM cells in sheep[24,31], and 16- to 64-cell stage embryos in cattle[2,32].

Contrary to this, it was suggested that eight-cell nuclei transferred to enucleated mouse zygotes can be reprogrammed to a certain extent, since the synthesis of 68/70 kD proteins that are characteristic of the first embryonic gene activity has resumed[8]. This was confirmed by Latham et al.[13] who reported that enucleated zygotes reconstituted with nuclei from germ lineage cells and four-cell stage mouse embryos were able to direct the synthesis of 70 kD complex following transcription and overnight culture to the two-cell stage. Furthermore, nuclei from late two-cell mouse embryos[25], and eight- and 16-cell rabbit embryos[16], transferred to enucleated zygotes could develop to the blastocyst stage. At present, however, no comparative observation has been reported concerning the developmental potential and developmental processes in relation to the nuclear reprogramming ability of oocytes and zygotes in the mouse.

The present study was undertaken to compare the developmental potential and developmental processes in the preimplantation period of enucleated oocytes and zygotes reconstituted with nuclei from late two-, four- and eight-cell stage mouse embryos.

MATERIALS AND METHODS

Collection of Oocytes and Embryos

F1 hybrid (C57BL/6J × CBA) and ICR strain females were superovulated with injection of 5 IU of pregnant mare's serum gonadotrophin (PMSG), followed 48 hr later by 5 IU of human chorionic gonadotrophin (hCG). Oocytes at metaphase II were collected from the ampullae of the oviducts of F1 females at 14–15 hr after hCG injection. Fertilized zygotes were collected from F1 females that were mated with the same strain of males at 22–23 hr after hCG injection. Cumulus cells were removed by treatment with 300 IU/ml hyaluronidase (Sigma, St. Louis, MO, USA). Late two-, four- and eight-cell embryos were collected by flushing the oviducts of ICR females which were mated with F1 strain males using M2 medium[6] at 44–45, 55–56 and 65–66 hr after hCG injection, respectively.

Enucleation of Oocytes and Zygotes

Manipulations were performed using an inverted microscope (Diaphot, Nikon) with Nomarski's contrast system and Narishige micromanipulators. Oocytes were enucle-

ated by removing the metaphase chromosomes arranged in the spindle of the second meiotic division. Oocytes were placed in a drop of M2 medium covered with paraffin oil. The zona pellucidae over the spindle area were slit with a fine glass needle along 10–20% of their circumference[28]. After zona slitting, the oocytes were placed in a drop of M2 medium containing 5 μg/ml cytochalasin B (CB) and 0.1 μg/ml colcemid. The oocytes were secured by a holding pipette opposite the slit in the zona. An enucleation pipette 20–25 μm in diameter with a beveled sharpened tip, was inserted into the perivitelline space through the slit and the spindle area of metaphase chromosomes was aspirated into the enucleation pipette with a small amount of cytoplasm (Fig. 1A). The extracted portions of the oocytes were stained with 1 μg/ml of Hoechst 33342 (Sigma) for 5 min and observed under fluorescence to confirm that the metaphase chromosomes had been removed correctly. Enucleation of zygotes was carried out by an undisruptive enucleation method described previously[14] (Fig. 1B).

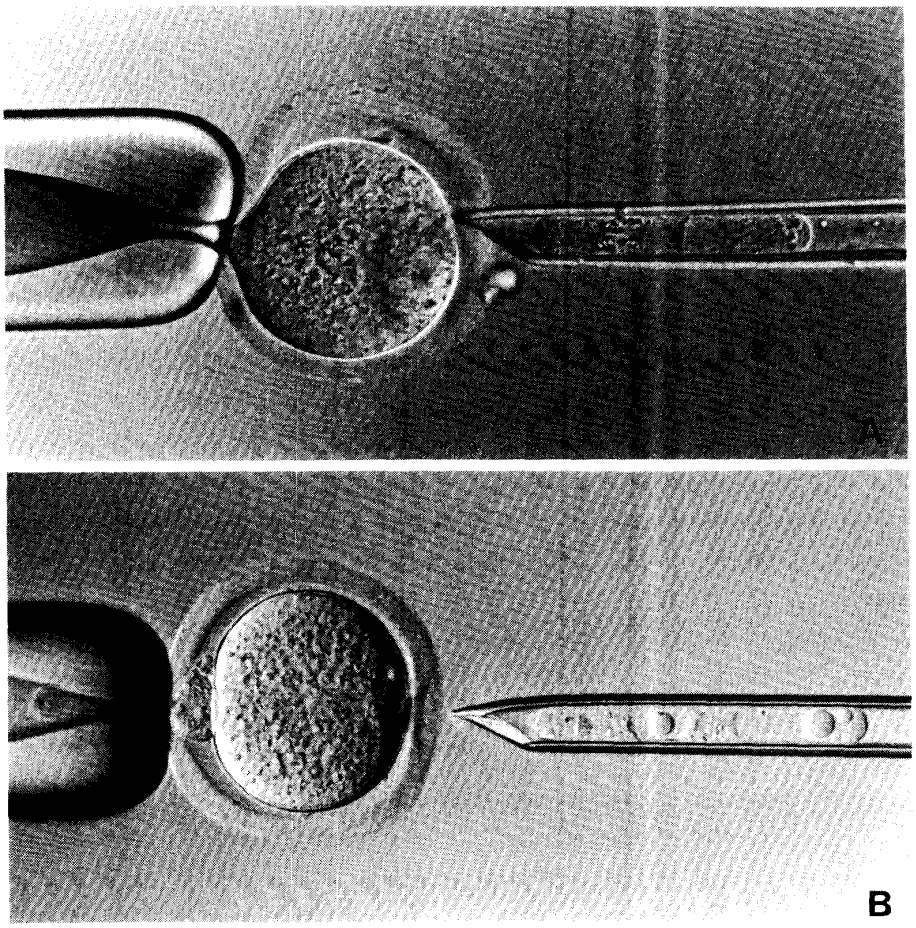

Fig. 1. Enucleation of recipient metaphase II oocyte (A) and zygote (B). ×340.

Nuclear Transplantation

Nuclear transplantation was carried out as described previously[14,15]. Immediately following enucleation of oocytes and zygotes, a karyoplast from late two–, four– and eight–cell donor embryos was introduced into the perivitelline space of the enucleated oocytes and zygotes. The manipulated eggs were then cultured in M16 medium[30] containing 100 μM EDTA in an atmosphere of 5% CO_2 in air at 37°C for 1 hr to allow close contact between the introduced karyoplast and the enucleated recipient cytoplasm.

Electrofusion and Activation

Membrane fusion was performed by electrofusion as described previously[3]. Manipulated eggs were placed in a 0.5 mm round wire stainless steel electrode chamber overlaid with 0.3 M mannitol containing 0.1 mM $MgSO_4$ and 0.05 mM $CaCl_2$. The eggs were aligned by exposure to an alternating current (AC) pulse of 0.6 MHz, 6V, for 6 sec, after which 3 to 5 direct current (DC) pulses of 1.5 kV/cm for 70 μsec, each pulse 1 sec apart, were applied to the chamber using an ELECTRIC CELL FUSION instrument (LF–100, Life Tec, Japan). At the end of the electrofusion procedure, eggs were placed in culture and checked every 10 min. Fusion occurred usually within 20–30 min. The fusion pulses also served to activate the oocytes.

In Vitro Culture, Monitoring and Embryo Transfer

After fusion, the reconstituted embryos were cultured in drops of M16 medium containing 100 μM EDTA, covered with paraffin oil, in an atmosphere of 5% CO_2 in air at 37°C. Embryos were monitored every 8 hr to assess activation and development. Nonenucleated zygotes and parthenogenetic diploid embryos which were created by exposure to electric fields, followed by treatment with 5 μg/ml CB for 5–6 hr were used as controls for the reconstituted zygotes and oocytes, respectively. Preimplantation development of parthenogenetic diploid embryos closely resembles that of fertilized ones, with all of the developmental events occurring on a time scale identical to that of fertilized embryos[9]. Reconstituted embryos and control nonenucleated zygotes that developed into blastocysts at 96 hr after culture were transferred to the uterine horn of Day–3 (2.5 days post coitum) pseudopregnant recipients (ICR strain).

Statistical Analysis

Data were analyzed by Chi–square test.

RESULTS

Efficiencies of Enucleation and Electrofusion

The metaphase II chromosomes and the pronuclei were successfully removed from 88.8% of oocytes and 95.7% of fertilized zygotes, respectively, although a significant difference ($P<0.01$) was observed between oocytes and zygotes (Table 1). Whereas 89.1% of zygotes fused with a karyoplast donor, 78.0% ($P<0.001$) of oocytes fused.

Table 1. The efficiencies of enucleation and electrofusion in the nuclear transfer procedure of mouse oocytes and zygotes[a]

Recipient cytoplasm	Donor nuclei	No. (%) of embryos				
		Operated	Enucleated	Injected	Fused	Activated[b]
Enucleated oocyte	2-cell	118	106(89.8)	103	82(79.6)	77(93.9)
	4-cell	76	66(86.8)	65	50(76.9)	46(92.0)
	8-cell	74	66(89.2)	64	49(76.6)	45(91.8)
	Total	268	238(88.8)[c]	232	181(78.0)[e]	168(92.8)
Enucleated zygote	2-cell	111	107(96.4)	105	94(89.5)	—
	4-cell	72	68(94.4)	67	60(89.6)	—
	8-cell	70	67(95.7)	66	58(87.9)	—
	Total	253	242(95.7)[d]	238	212(89.1)[f]	—

[a] Results were pooled from 4 to 6 replicates.
[b] Based on the number of fused embryos.
[c,d] Values with different superscripts are significantly different ($P<0.01$).
[e,f] Values with different superscripts are significantly different ($P<0.001$).

The proportion of oocytes that were activated following electrofusion treatment and exhibited the extrusion of extra polar body or nuclear swelling was 92.8%. The efficiency of oocyte activation was confirmed when nonenucleated oocytes were submitted to the same electrofusion treatment. Forty seven (90.4%) of 52 nonenucleated oocytes were activated.

Development In Vitro

In vitro development of reconstituted embryos to the blastocyst stage was affected by the stage of donor nuclei but not by different cytoplasms as shown in Table 2. Development to the blastocyst stage was obtained from enucleated oocytes (28.0%) and zygotes (30.9%) reconstituted with nuclei of two-cell stage embryos. When nuclei from four- and eight-cell stage embryos were transferred to enucleated oocytes and zygotes, 28.6 to 68.3% of reconstituted embryos cleaved once or twice, however, none developed into blastocysts. On the other hand, all of the nonenucleated zygotes and 83.7% of parthenogenetic diploid embryos developed to the blastocyst stage.

Time Course for Development In Vitro

The times of cleavage and blastocoele formation of reconstituted embryos were compared with those of corresponding control embryos (Fig. 2). No Significant difference was found, at any period after manipulation, between the cleavage rates of reconstituted zygotes and control nonenucleated zygotes. Although there were no significant differences at 8 and 16 hr after manipulation, reconstituted oocytes had a significantly lower ($P<0.01$) cleavage rate at 24 hr after manipulation when compared

Table 2. In vitro development of nuclear transplant mouse embryos

Recipient cytoplasm	Donor nuclei	No. of embryos cultured	No. (%) of embryos developed to		
			2-cell	4-cell	Blastocyst
Enucleated	2-cell	82	66(80.5) [a]	36(43.9) [a]	23(28.0) [a]
oocyte	4-cell	50	21(42.0) [b]	0(0.0) [b]	0(0.0) [b]
	8-cell	49	14(28.6) [b]	1(2.0) [b]	0(0.0) [b]
Nonenucleated parthenogenone	—	43	43(100.0) [c]	40(93.0) [c]	36(83.7) [c]
Enucleated	2-cell	94	79(84.0) [a]	44(46.8) [a]	29(30.9) [a]
zygote	4-cell	60	41(68.3) [d]	2(3.3) [b]	0(0.0) [b]
	8-cell	58	35(60.3) [d]	0(0.0) [b]	0(0.0) [b]
Nonenucleated zygote	—	46	46(100.0) [c]	46(100.0) [c]	46(100.0) [d]

[a, b, c, d] Values with different superscripts in the same column are significantly different ($P<0.05$).

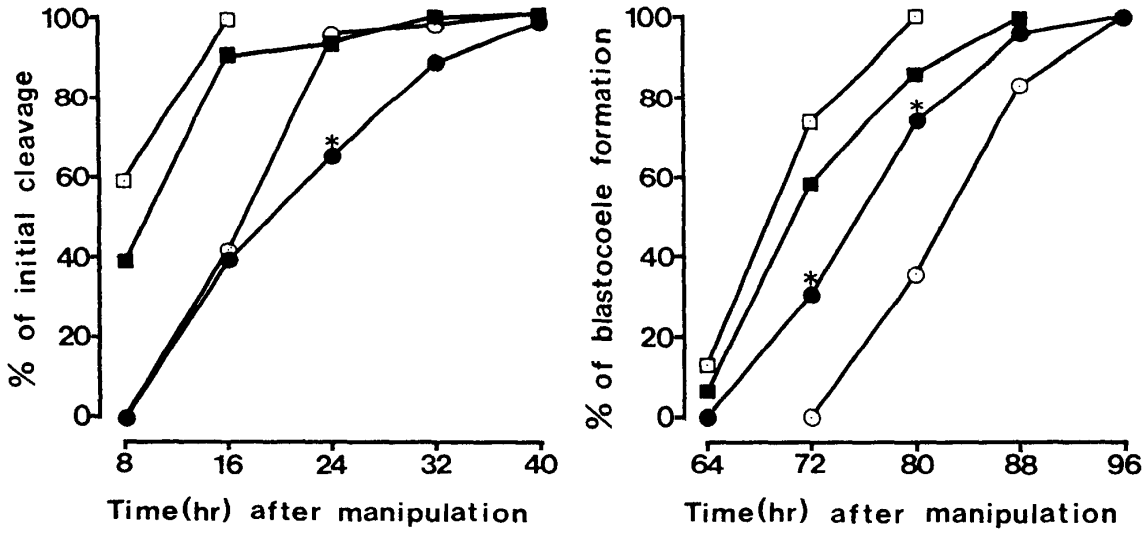

Fig. 2. Proportions of initial cleavage and blastocoele formation per unit time of control and nuclear transplant mouse embryos. ●——● Reconstituted oocytes receiving nuclei from two-cell embryos. ○——○ Nonenucleated diploid parthenogenones as control for reconstituted oocytes. ■——■ Reconstituted zygotes receiving nuclei from two-cell embryos. □——□ Nonenucleated zygotes as control for reconstituted zygotes. *Significantly different from the corresponding controls at the same time after manipulation ($P<0.01$).

to the nonenucleated diploid parthenogenones.

Blastocoele formation rates of reconstituted oocytes were significantly higher ($P<0.01$) than those of nonenuleated diploid parthenogenones at 72 and 80 hr after manipulation. There were no significant differences, at any period after manipulation, between reconstituted zygotes and control nonenucleated zygotes.

Development In Vivo

After transfer to recipient females of blastocysts derived from reconstituted oocytes and zygotes with a nucleus from late two-cell embryos, 9.1 and 11.5% gave rise to live young, respectively (Table 3). Of 36 blastocysts derived from control nonenucleated zygotes, 21 (58.3%) live young were born.

Table 3. In vivo development of nuclear transplant mouse embryos

Recipient cytoplasm	Donor nuclei	No. pregnant / No. of recipient used (%)	No. of young / No. of embryos transferred (%)
Enucleated oocyte	2-cell	2/6(33.3)	2/22(9.1) [a]
Enucleated zygote	2-cell	3/6(50.0)	3/26(11.5) [a]
Nonenucleated zygote	—	4/5(80.0)	21/36(58.3) [b]

[a, b] Values with different superscripts are significantly different ($P<0.01$).

Discussion

When the first polar body is used as a reference point to estimate the location of the chromosomes, enucleation is not reliable[4,18,21,24] since metaphase II oocytes had their chromosomes located separately from the first polar body[11] or the first polar body may be degenerated[16]. In the present study, successful enucleation of the oocytes at metaphase II was achieved using the interference microscope without any staining. The metaphase II chromosomes with the spindle were easily aspirated into the enucleation pipette when the zona pellucidae over the spindle area were slit before enuceation. High enucleation rates have also been obtained from telophase oocytes of first meiotic division[11] and recently ovulated metaphase oocytes [26] when the first polar body and spindle area were removed.

Electrofusion of nuclear transplant oocytes has an advantage in that activation of oocytes is induced with electrofusion pulses. Therefore, further activation of oocytes may be unnecessary. In this study, high activation rates were obtained in enucleated and nonenucleated oocytes with electrofusion pulses. However, the fusion rate of

nuclear transplant oocytes was lower compared to nuclear transplant zygotes when the same fusion pulses were applied. The difference in fusion rate between nuclear transplant oocytes and zygotes may be due to the different plasma membrane structures of the oocyte and zygote. Fertilization and activation of oocytes can lead to plasma membrane changes, such as cortical granule release and disappearance of microvilli[7,10]. Electrofusion and activation rates of nuclear transplant oocytes can be also affected by oocyte age[4].

In the present study, live young were produced from both reconstituted oocytes and zygotes that received a nucleus from late two–cell embryos. Full-term development from enucleated oocytes reconstituted with late two-cell nuclei was also reported in a previous study[11]. Although development to the blastocyst stage was achieved from enucleated oocytes reconsitituted with ICM cell stage nuclei[11,27], both enucleated oocytes[11,27], and zygotes[15,29] could not support development of nuclei from eight-cell embryos. It is suggested that enucleated zygotes as well as oocytes can support development to the blastocyst stage and full term at least, of nuclei from embryos around the onset of transcriptional activity. In the mouse, the transcriptional activity of the embryonic genome occurs at the two-cell stage[1,17]. Similar evidence was obtained from a nuclear transfer experiment in rabbits[16], in which development to the blastocyst stage was achieved from both enucleated oocytes and zygotes reconstituted nuclei derived from eight- and 16-cell stage embryos around the onset of transcriptional activity. However, there has been insufficient investigations done in sheep, pigs and cattle. It appears that more detailed experimental data on the development of nuclear transferred zygotes considering cell cycle stage[25], are needed to estimate the ability of enucleated zygotes to reprogram the transferred nuclei.

The timing of the occurrence of developmental events such as cleavage, compaction and blastocoele formation is an important parameter in evaluation of the reprogramming of the donor nucleus. However, few observations have been made of the developmental events in reconstituted oocytes and zygotes. Developmental processes of some reconstituted embryos especially those without polar body, were characterized by delayed cleavage at the end of the first cell cycle and earlier blastocoele formation compared to those of the control diploid parthenogenones. After the first cleavage, their cell divisions progressed with a short period of the cell cycle to the blastocyst stage (data not shown). These abnormalities in the developmental processes could be due to the failure in the complete reprogramming of the donor nucleus. It has been suggested that the reconstituted embryos that did not extrude a polar body have a tetraploid chromosome constitution as a result of the failure in reprogramming[12], which presumably affect the developmental processes of the reconstituted embryos. Delayed development was also observed in some reconstituted zygotes, while most the reconstituted zygotes cleaved and formed blastocoeles at around the same time as observed in the nonenucleated zygote controls.

In conclusion, the results of this study show that enucleated zygotes as well as oocytes can support development to the blastocyte stage and to term of nuclei introduced from late two-cell embryos.

ACKNOWLEDGMENTS

We wish to thank Dr. A. A. Rayos for this helpful evaluation of the manuscript. This study was supported by Grants-in-Aid for Scientific Research (No. 02556039) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1) BENSAUDE, O., BABINET, C., MORANGE, M. & JACOB, F. (1983): Heat shock proteins, first major products of zygotic gene activity in the mouse embryo. *Nature*, **305**, 331–333
2) BONDIOLI, K. R., WESTHUSIN, M. E. & LOONEY, C. R. (1990): Production of identical bovine offspring by nuclear transfer. *Theriogenology*, **33**, 165–174
3) CHEONG, H. T., TANIGUCHI, T., HISHINUMA, M., TAKAHASHI, Y. & KANAGAWA, (1991): Effects of various electric fields on the fusion and in vitro development of mouse two-cell embryos. *Theriogenology*, **36**, 875–885
4) COLLAS, P. & ROBL, J. M. (1990): Factors affecting the efficiency of nuclear transplantation in the rabbit embryos. *Biol. Reprod.*, **43**, 877–884
5) COLLAS, P. & ROBL, J. M. (1991): Relationship between nuclear remodeling and development in nuclear transplant rabbit embryos. *Biol. Reprod.*, **45**, 445–465
6) FULTON, B. P. & WHITTINGHAM, D. G. (1978): Activation of mammalian oocytes by intracellular injection of calcium. *Nature*, **273**, 149–151
7) GULYAS, B. J. & YUAN, L. C. (1985): Cortical reaction and zona hardening in mouse oocytes following exposure to ethanol. *J. Exp. Zool.*, **233**, 269–276
8) HOWLETT, S. K., BARTON, S. C. & SURANI, M. A. H. (1987): Nuclear cytoplasmic interactions following nuclear transplantation in mouse embryos. *Development*, **101**, 915–923
9) HOWLETT, S. K., BARTON, S. C. & SURANI, M. A. H. (1989): Effects of the number, stage and parental origin of nuclei in reconstructed mouse eggs. *J. Reprod. Fertil. (Suppl.)*, **38**, 99–105
10) HYTTEL, P., GREVE, T. & CALLESEN, H. (1989): Ultrastructural aspects of oocyte maturation and fertilization in cattle. *J. Repord. Fertil. (Suppl.)*, **38**, 35–47
11) KONO, T., KWON, O. Y. & NAKAHARA, T. (1991): Development of enucleated mouse oocytes reconstituted with embryonic nuclei. *J. Reprod. Fertil.*, **93**, 165–172
12) KONO, T., KWON, O. Y., WATANABE, T. & NAKAHARA, T. (1992): Development of mouse enucleated oocytes receiving a nucleus from different stage of the second cell cycle. *J. Repord. Fertil.*, **94**, 481–487
13) LATHAM, K. E., SOLTER, D. & SCHULTZ, R. M. (1991): Activation of a two-cell stage-specific gene following trasfer of heterologous nuclei into enucleated mouse embryos. *Mol. Repord. Dev.*, **30**, 182–186

14) MCGRATH, J. & SOLTER, D. (1983): Nuclear transplantation in the mouse embro by microsurgery and cell fusion. *Science*, **220**, 1300–1302
15) MCGRATH, J. & SOLTER, D. (1984): Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. *Science*, **226**, 1317–1319
16) MODLINSKI, J. A. & SMORAG, Z. (1991): Preimplantation development of rabbit embryos after transfer of embryonic nuclei into different cytoplasmic environment. *Mol. Reprod. Dev.*, **28**, 361–372
17) PIKO, L. & CLEGG, K. B. (1982): Quantitative changes in total RNA, total poly(A), and ribosomes in early mouse embryos. *Dev. Biol.*, **89**, 362–378
18) PRATHER, R. S., BARNES, F. L., SIMS, M. M., ROBL, J. M., EYESTONE, W. H. & FIRST, N. L. (1987): Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. *Biol. Repord.*, **37**, 859–866
19) PRATHER, R. S. & FIRST, N. L. (1990a): Cloning embryos by nuclear transfer. *J. Reprod. Fertil. (Suppl.)*, **41**, 125–134
20) PRATHER, R. S. & FIRST, N. L. (1990b): Nuclear transfer in mammalian embryos. *Int. Rev. Cytol.*, **120**, 169–190
21) PRATHER, R. S., SIMS, M. M. & FIRST, N. L. (1989): Nuclear transplantation in early pig embryos. *Biol. Reprod.*, **41**, 414–418
22) ROBL, J. M., PRATHER, R., BARNES, F., EYESTONE, W., NORTHEY, D., GILLIGAN, B. & First, N. L. (1987): Nuclear transplantation in bovine embryos. *J. Anim. Sci.*, **67**, 642–647
23) ROBL, J. M. & STICE, S. L. (1989): Prospects for the commercial cloning of animals by nuclear transplantation. *Theriogenology*, **31**, 75–84
24) SMITH, L. C. & WILMUT, I. (1989): Influence of nuclear and cytoplasmic activity on the development in vivo of sheep embryos after nuclear transplantation. *Biol. Reprod.*, **40**, 1027–1035
25) SMITH, L. C., WILMUT, I. & HUNTER, R. H. F. (1988): Influence of cell cycle stage at nuclear transplantation on the development in vitro of mouse embryos. *J. Reprod. Fertil.*, **84**, 619–624
26) STICE, S. L. & ROBL, J. M. (1988): Nuclear reprogramming in nuclear transplant rabbit embryos. *Biol. Reprod.*, **39**, 657–664
27) TSUNODA, Y., TOKUNAGA, T., IMAI, H. & UCHIDA, T. (1989): Nuclear transplantation of male primordial germ cells in the mouse. *Development*, **107**, 407–411
28) TSUNODA, Y., YASUI, T., NAKAMURA, K., UCHIDA, T. & SUGIE, T. (1986): Effect of cutting the zona pellucida on the pronuclear transplantation in the mouse. *J. Exp. Zool.*, **240**, 119–125
29) TSUNODA, Y., YASUI, T., SHIODA, Y., NAKAMURA, K., UCHIDA, T. & SUGIE, T. (1987): Full-term development of mouse blastomere nuclei transplanted into enucleated two-cell embryos. *J. Exp. Zool.*, **242**, 147–151
30) WHITTINGHAM, D. G. (1971): Culture of mouse ova. *J. Reprod. Fertil.* (Suppl.), **14**, 7–21
31) WILLADSEN, S. M. (1986): Nuclear transplantation in sheep embryos. *Nature*, **320**, 63–65

32) WILLADSEN, S. M., JANZEN, R. E., MCALISTER, R. J., SHEA, B. F., HAMILTON, G. & MCDERMAND, D. (1991): The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. *Theriogenology*, **35**, 161–170